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[12] **Description of Patent Invention**

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1 page of Claims, 6 pages of Description, and 1 page of Drawings

54] Name of the invention: Method for L-homocysteine assay and reagents thereof

[57] Abstract

The present invention relates to an enzymatic recycling amplifying technique and reagents thereof, which can be used to detect or quantitatively determine L-homocysteine in body fluid samples. Its characteristics are, to increase the sensitivity of assay by using the recycling amplifying technique, so the L-homocysteine from body fluid samples can be measured automatically just like using routine enzymatic detection reagents. Through the enzymatic cycle consisting of L-homocysteine methyltransferase and L-homocysteine hydrolase, the L-homocysteine in the body fluid samples reacts repetitively with S-adenosyl-L-methionine to form adenosine. The rate of the adenosine production is in proportion to the L-homocysteine content in samples, thus L-homocysteine content in body fluid samples can be detected by detecting the rate of adenosine production. By using the method and quantitative reagents provided in the present invention, L-homocysteine in samples can be detected conveniently, rapidly, automatically and with higher sensitivity.

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Claims

What is claimed is:

1. A method and reagents for detecting or determining L-homocysteine in body fluid samples, its characteristic lies in:

(a) Through the enzymatic cyclic reaction consisting of L-homocysteine S-methyltransferase (EC 2.1.1.10), adenosylhomocysteinase (EC 3.3.1.1) and their substrate, the L-homocysteine in samples is catalyzed to produce adenosine. (b) The synthetic rate of adenosine is in proportion to the L-homocysteine content in a sample. By determining the adenosine content, the L-homocysteine content in a sample can be calculated.

2. The method of claim 1, wherein the L-homocysteine-converting enzyme in the enzymatic reactions is S-adenosyl-L-methionine: L-homocysteine methyltransferase (EC 2.1.1.10) and S-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1). The co-substrate in the enzymatic reactions is S-adenosyl-L-methionine, which is available commercially or obtained through other enzymatic reactions.

3. The enzymatic cyclic reaction of claim 1, further includes a thiol-selective reductive reagent, such as 1, 4-Dithiothreitol (DTT); monovalent and divalent metal ions, such as potassium ion (K^+), magnesium ion (Mg^{2+}).

4. The method of claim 1, wherein the L-homocysteine content is measured by detecting or determining the adenosine content. The adenosine detection is based on a chromogenic reaction of the hydrogen peroxide (Trinder's method), in which the reagents include the enzymes and co-substrate of claim 2, further include adenosine deaminase, purine nucleoside phosphorylase, xanthine oxidase, peroxidase, and Trinder's chromogens reagents, etc.

5. The method of claim 1, wherein the L-homocysteine content is measured by detecting or determining the adenosine content. The adenosine detection is based on the ammonia detection method, in which the reagents include the enzymes and co-substrate of claim 2, further include adenosine deaminase, glutamatedehydrogenase, a-ketoglutarate, reduced coenzyme I or Π or their analogues thereof, etc.

6. The method of claim 2, wherein the enzymatic reactions is to produce S-adenosyl-L-methionine. The reaction system is composed of adenosine triphosphate, methionine and adenosine triphosphate: L-methionine S-adenosyltransferase (ATP: L-methionine S-adenosyltransferase, EC 2.5.1.6).

Description

Field of the invention

The present invention relates to a method for measuring L-homocysteine content and reagents thereof.

Background of the invention

The L-homocysteine is a thiol-containing amino acid produced by the demethylation of methionine in cell. Recent research shows, through forming the peroxide and hyperoxide, L-homocysteine can damage vascular endothelial cell, change the function of blood clotting factor, increases risk of thrombosis, promote atherosclerotic diseases, increase the incidence and mortality of cardiovascular disease. Therefore, the method to detect the L-homocysteine concentration in blood samples is significantly important in clinical application. When L-homocysteine accumulates in cell or transport to the blood cycle system, most of L-homocysteine is present in form of oxidized L-homocysteine and bind to other plasma proteins via disulfide bonds. The content of reduced L-homocysteine accounts for 1% in blood. To measure the L-homocysteine content, generally, the oxidated L-homocysteine is reduced to be as reduced L-homocysteine using reducing reagents.

The main clinical detection method of L-homocysteine content includes: high performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), fluorescence polarization immunoassay (FPLA). The above-mentioned methods require specific apparatus, complex procedure, and they are also time-consuming and much expensive. So these methods are not applicable to the high-throughout clinical detection automatically.

Due to the low concentration of L-homocysteine in samples, the routine clinical enzymatic assays can not meet the sensitivity required, which limits their applications. According to the routine enzymatic detection techniques, Naoto Matsuyama et al. (refer to U.S.A. patent) developed a noncyclic enzymatic method employing L-homocysteine S-methyltransferase and adenosylhomocysteinase to enhance the detection sensitivity. But the procedure of this method is tedious, the reagents used is complex and it is necessary to add mercapto compounds before detection, and it is also necessary to carry out several simultaneous blank control tests during the detection, so this method increase the inaccuracy.

The present invention claimes a novel detection method of L-homocysteine content in body fluid. This method alleviates the interference caused by the interfering chemical existing in body fluid, which therefore enhance the detection sensitivity extremely. In the present invention, the enzymes involving in the enzymatic reaction refers to those are frequently used to detect the L-homocysteine content, such as L-homocysteine S-methyltransferase and S-adenosylhomocysteine hydrolase, herein they are applied to the enzymatic recycling

amplifying technique for the first time. Further, the present invention provides, for the first time, a method of enzymatic cyclic reaction employing two enzymes above-mentioned, and the L-homocysteine detection reagents prepared using this method. The L-homocysteine detection reagents can be applied to the wide-scale clinical automatic analyzer apparatus, and satisfy the request of determination of large-scale samples.

Detailed description of the invention

The present invention provides as a method and reagents thereof for assessing L-homocysteine content in body fluid, this method employ the enzymatic recycling amplifying technique, which mainly consists of L-homocysteine S-methyltransferase and adenosylhomocysteinase. The oxidized L-homocysteine in body fluid samples is reduced by reducing reagent, and reacts with S-adenosyl-L-methionine to produce S-adenosyl-L-homocysteine and L-methionine, this step is catalyzed by the L-homocysteine methyltransferase (EC 2.1.1.10). Subsequently, S-adenosyl-L-homocysteine is hydrolyzed to form L-homocysteine and adenosine, which is catalyzed by S-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1) (shown in Figure). Therefore, utilizing the L-homocysteine in samples, the enzymatic cyclic reaction can produce the adenosine consecutively. The rate of adenosine production is in proportion to the L-homocysteine content in samples, thus the L-homocysteine content in body fluid samples can be detected by detecting the rate of adenosine production.

There are many methods for adenosine measurement, the present invention only lists two routine auxiliary enzyme systems: (1) With adenosine deaminase, adenosine forms ammonia and inosine, the latter reacts with phosphoric acid to form the hypoxanthine which is catalyzed by the purine nucleoside phosphorylase. In presence of xanthine oxidase, hypoxanthine is oxidized finally to form uric acid, and simultaneously produce hydrogen peroxide. Hydrogen peroxide reacts with the chromogenic reagent to produce detectable pigments. (2) Using other ammonia assay methods, for an example, glutamatedehydrogenase reaction system. In presence of glutamatedehydrogenase, ammonia reacts with a-ketoglutarate to form L-glutamate, and NADH (or NADPH) is oxidized to NAD (or NADP). The decrease absorption of the reaction system can be monitored at 340nm. The sensitivity of the detection increase extremely due to the consecutive production of adenosine and the recycling of L-homocysteine in samples.

Figure legends:

The principle of the present invention lies in: with L-homocysteine methyltransferase (HcyMetase), the L-homocysteine in samples is reduced by the reducing reagent, then reacts with S-adenosyl-L-methionine (AdoMet) to form S-adenosyl-L-homocysteine (AdoHcy) and L-methionine. In presence of S-adenosyl-L-homocysteine hydrolase (AdoHcyase), S-adenosyl-L-homocysteine is hydrolyzed to form L-homocysteine and adenosine (Ado). In

the reaction system, the L-methionine reacts with adenosine triphosphate (ATP) to form S-adenosyl-L-methionine (AdoMet) in presence of L-methionine-S-adenosyltransferase (MAT). The L-homocysteine in samples is recycled and adenosine is produced consecutively. The rate of adenosine production is in proportion to the L-homocysteine content in samples. Accordingly, based on the rate of the adenosine production, the L-homocysteine content in body fluid samples can be determined.

Most L-homocysteine in blood is present in form of oxidized L-homocysteine, among which 80-90% binds to protein, 5-10% binds to L-homocysteine itself, and 5-10% binds to cysteine to form cysteine-homocysteine complex disulfate compounds. The reduced L-homocysteine only accounts for about 1%. Before detecting the total L-homocysteine content, the reductant is used to reduce the disulfide bonds, and the oxidized L-homocysteine is reduced to the reduced L-homocysteine. The most commonly used reductant includes 1,4-Dithiothreitol (DTT), Tris (2-carboxyethyl), phosphine (TCEP), etc. The concentration of reductant in the reaction is not supposed to be too high in order to avoid interfering with the reaction. In a preferred embodiment the concentration of DTT is less than 10mM.

Additionally, the thiol-containing reductant is also used as an active agent of L-homocysteine methyltransferase and L-methionine S-adenosyltransferase.

The initial compound of the enzymatic cyclic reaction is S-adenosyl-L-methionine which is now commercially available. However, because the common commodity product is unstable extremely, in the present invention, S-adenosyl-L-methionine is produced by introducing an auxiliary enzyme system that synthesizes S-adenosyl-L-methionine with L-methionine and ATP in the presence of L-methionine-S-adenosyltransferase. An advantage of introducing this auxiliary enzyme system is to shift the reaction balance and steer the main cyclic reaction in the desired direction. Further, it is useful to decrease the amount of the L-methionine used in the detection. It is not essentially necessary in this method to introduce this auxiliary enzyme system, because all kinds of enzyme systems or methods that can produce and supply enough and constant S-adenosyl-L-methionine are feasible for the present invention.

The present invention provides, for the first time, an L-homocysteine detection method employing the enzymatic reaction cycle comprising of L-homocysteine-S- methyltransferase and adenosylhomocysteinase. Due to the advantages of the enzymatic recycling amplifying technique, the higher amount of enzyme used in the enzymatic cyclic reaction is not essentially necessary. More preferably, the final concentration of enzyme should be between 0.1~50ku/L, and most preferably between 0.2~10ku/L. The higher amount of enzyme will not affect the accuracy and consistence of the detection method, but it will increase the cost of reagents. Optimizing of the amount of enzyme and cosubstrate will shift the cyclic reaction in the desired direction. The amount of ATP should be excessive and also much higher than the amount of L-methionine in the reaction system. Preferably, the concentration of ATP should usually be between 0.1-90mM/L, and more preferably between 1- 80mM/L. The amount of S-adenosyl-L-homocysteine hydrolase should be higher than the amount of L-homocysteine S-methyltransferase. Preferably, the amount of L-methionine

S-adenosyltransferase should be higher than the amount of L-homocysteine S-methyltransferase. Furthermore, the amount of adenosine deaminase should be excessive.

The present invention provides a detection method, which is not interfered by the interfering compound in body fluid samples, such as adenosine, L-methionine, cystathionine, etc.

The present invention provides, for the first time, two detection reagents comprising of L-homocysteine methyltransferase and S-adenosyl-L-homocysteine hydrolase, which forms an enzyme reaction cycle. The detection reagents are specially designed to detect the L-homocysteine content in body fluid samples.

According to the enzymatic cyclic reaction described in the present invention, all kinds of L-homocysteine detection reagent kits can be prepared according to routine enzymatic detection techniques. In a more preferable embodiment, the method of the present invention includes the peroxidase spectrophotometric method (also called the Trinder's method). The enzymatic cyclic reaction produces adenosine, which is catalyzed subsequently by adenosine deaminase, purine nucleoside phosphorylase, xanthine oxidase, and finally forms hydrogen peroxide. In presence of hydrogen peroxidase, hydrogen peroxide reacts with phenols-4-amino-antipyrine and oxidative chromogenic reagent to form pigments. The L-homocysteine content in a sample can be calculated based on the rate of pigment production. There are many oxidative chromogenic reagent used for the clinical enzymatic detection reagent, such as N-ethyl-N-(2-hydroxy -3-sulfopropyl)-3-methylaniline (TOOS), 2,4,6-Tribromo-3-hydroxybenzoic acid (TBHBA), N-ethyl-N-(3-sulfopropyl)-3-methoxyaniline (ADPS) N-Ethyl-N- (2-hydroxy-3-sulfopropyl)-3-methoxylaniline (ADOS), N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (DAOS), etc. Another class of oxidative chromogenic reagent doesn't need to couple with 4-aminoantipyrine (4-AA). More preferably, the concentration of oxidative chromogenic reagent is between 0.01-10 mM/L, and most preferably between 0.5-3 mM/L.

In another more preferable embodiment, includes the enzymatic method to detect ammonia. The enzymatic cyclic reaction produces adenosine, which can be converted to ammonia with adenosine deaminase. Reduced coenzyme (such as NADP, NADPH, thio-NADP, thio-NADPH, etc.) can be further oxidized by the auxiliary system of glutamatedehydrogenase. By monitoring the decrease rate of absorption at 340nm, the L-homocysteine content in a sample can be calculated. More preferably, the concentration of reduced coenzyme is between 0.1-0.8mM, and most preferably between 0.15-0.4mM/L.

The L-homocysteine detection reagent prepared according to the enzymatic reaction mechanism described in the present invention, should include the buffer, such as phosphate buffer, N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES) buffer, etc.; metal ion, such as magnesium ion; and other necessary component for the reaction. It also includes surface active agent, chelant (for example, EDTA) and antiseptic, etc.

The two detection methods mentioned above are the same with the method routinely used in the art to which this invention belongs, such as using the endpoint method or the rate method, the content in samples can be calculated according to the standard sample or the standard

concentration curve. These methods will not be described in details herein. Those skilled in the art to which this invention belongs can prepare analogue reagents according to the principle and method provided in the present invention.

Examples:

Example 1:

Through the enzymatic cyclic reaction consisting of L-homocysteine S-methyltransferase and S-adenosyl-L-homocysteine hydrolase, hydrogen peroxide can be produced. Hydrogen peroxide reacts with the chromogens reagents to form pigments (Trinder's reaction).

Reagent 1: (R1:R2=3:1)

Reagent composition	The amount/ liter	The range of amount /liter
Phosphate Buffer Saline, pH7.0, 37°C	100 mM	100-300 mM
EDTA.2Na	0.2 mM	0.1-20 mM
$MgSO_4$	15 mM	0.5-100 mM
Triton X-100	0.1%	0.01-5%
DTT	2 mM	0.1-20 mM
ATP	80 mM	0.1-90 mM
ADA	3 ku	0.1-200 ku
ADPS	2 mM	0.01-20 mM
AdoHcyase	10 ku	0.1-50 ku
D-mannitol	20 mM	1-100 mM
Oxidized coenzyme NAD	0.1 mM	0.01-20 mM
L-methionine S-adenosyltransferase	15 ku	0.1-50 ku
Purine nucleoside phosphorylase	2 ku	0.1-50 ku
Xanthine oxidase	3 ku	0.1-50 ku
Horseradish peroxidase	2 ku	0.1-50 ku
L-homocysteine methyltransferase	4 ku	0.1-100 ku

Reagent 2: (R1:R2=3:1)

Reagent composition	The amount/liter	The range of amount/liter
Phosphate buffer saline, pH7.0, 37°C	100 mM	100-300 mM
EDTA.2Na	0.2 mM	0.1-20 mM
$MgSO_4$	15 mM	0.5-100 mM
Triton X-100	0.1%	0.01-5%
L-methionine	0.5 mM	0.1-90 mM
4-Aminoantipyrine	1 mM	

The reagent 1 in example 1 is used to reduce the oxidized L-homocysteine and eliminate the interference caused by the adenosine in samples. Meanwhile, the reagent 1 will form an enzymatic cyclic reaction, which is helpful to eliminate the side reaction. Fixed-time method is employed to detect the sample. The ratio between reagent and sample is not definite strictly, but it should be the same among a same batch of samples. In a preferred embodiment, the ratio of reagent 1: sample: reagent 2 is 300:20:100, the reaction temperature is 37°C, the detection wavelength is 540nm. After the reagent 1 is mixed with a sample or standard sample, the mixture should be incubated at the test temperature for 300 seconds, which can eliminate the interference caused by adenosine in samples. After the reagent 2 is added, delay duration of 0-120 seconds is necessary before detection, which can eliminate the side reaction caused by the S-adenosyl-L-homocysteine converted from S-adenosyl-L-methionine. The detection time maybe up to 180-200 seconds, and reliable measure value should be obtained at least at two time points during detection.

Example 2

Through the enzymatic cyclic reaction comprising of L-homocysteine S-methyltransferase and S-adenosyl-L-homocysteine hydrolase, the reduced coenzyme can be oxidized to the oxidized coenzyme. The rate of the oxidative reaction of coenzyme can be detected at fixed-time point by using the ammonia assay method.

Reagent 1: (R1:R2=3:1)

Reagent composition	The amount /liter	The range of amount/liter
Phosphate buffer saline, pH7.0, 37°C	150 mM	500-500 mM
EDTA.2Na	0.5 mM	0.1-20 mM
$MgSO_4$	15 mM	0.5-100 mM
DTT	1.5 mM	0. 1-20 mM
ATP	80 mM	0.1-90 mM
ADA	3 ku	0.1-200 ku
AdoHcyase	10 ku	0.1-50 ku
Albumin Bovine	0.2%	0.01-10%
a-Ketoglutarate	7.5 mM	1-20 mM
L-methionine S-adenosyltransferase	15 ku	0.1-100 ku
Glutamatedehydrogenase	5 ku	1-50 ku
L-homocysteine methyltransferase	5 ku	0.1-50 ku

Reagent 2: (R1:R2=4:1)

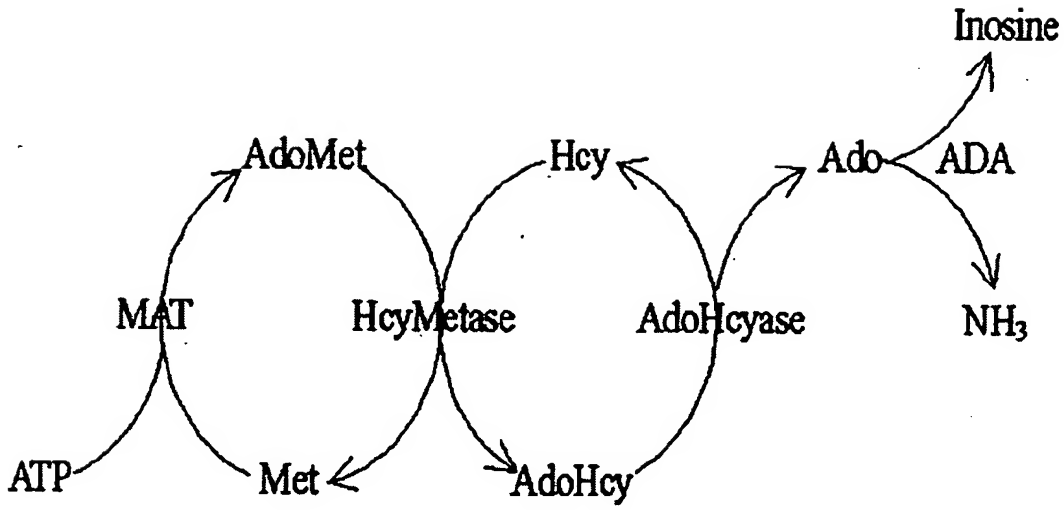
Reagent composition	The amount /liter	The range of amount /liter
HEPES buffer, pH8.3, 37°C	50 mM	10-200 mM
EDTA.2Na	0.5 mM	0.1-20 mM
$MgSO_4$	15 mM	0.5-100 mM
L-methionine	10 mM	0.1-90 mM
D-mannitol	20 mM	1-100 mM
Reduced coenzyme NADPH	0.8 mM	0.1-10 mM

Lactate dehydrogenase	2 ku	0.5-20 ku

The reagent 1 in example 2 is used to reduce the oxidized L-homocysteine. The whole enzymatic cyclic reaction reagent is composed of the reagent 1 in example 2 and the reagent 2 in example 2. Fixed-time method is employed to detect the sample. The ratio between reagent and sample is not definite strictly, but it should be the same among the same batch of samples. In a preferred embodiment, the ratio of reagent 1: sample: reagent 2 is 200:25:50, the reaction temperature is 37°C, the detection wavelength is 340nm. After the reagent 1 is mixed with a sample or standard sample, the mixture is incubated at the test temperature for 300 seconds. Then the reagent 2 is added. A delay time of 60-120 seconds is necessary before detection, which can eliminate the side reaction caused by ammonia, pyruvic acid, or S-adenosyl-L-homocysteine converted from S-adenosyl-L-methionine. The detection time maybe up to 180 seconds, and reliable measure value should be obtained at least at two time points during detection.

The above-mentioned examples are offered by way of illustration, not by way of limitation. Indeed, various modifications of the present invention, in addition to those described herein, will become apparent to those skilled in the art to which this invention belongs. Such assays modified or the like are intended to fall within the scope of the appended claims.

The figure


Inosine
AdoMet
Hcy
Ado
ADA
MAT
HcyMetase
AdoHcyase
NH3
ATP
Met
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